AD_____

Award Number: DAMD17-03-1-0707

TITLE: BRCC36, A Novel Subunit of a BRCA1/2 E3 Ubiquitin Ligase Complex: Candidate Breast Cancer Susceptibility Gene

PRINCIPAL INVESTIGATOR: Andrew K. Godwin, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111-2412

REPORT DATE: September 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2004	Annual (1 Sep 2003 - 31 Aug 2004)

4. TITLE AND SUBTITLE
BRCC36, A Novel Subunit of a BRCA1/2 E3 Ubiquitin Ligase Complex: Candidate Breast Cancer Susceptibility Gene

5. FUNDING NUMBERS
DAMD17-03-1-0707

6. AUTHOR(S)
Andrew K. Godwin, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111-2412

E-Mail: Andrew.godwin@fccc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Besides family history of cancer and an individual's age, no single etiologic factor can identify women at an increased risk for the disease. Approximately 10% of all cases of breast cancer exhibit a familial pattern of incidence. Efforts to identify the genetic basis of familial breast cancer reached fruition some years ago, when the breast-cancer susceptibility genes, BRCA1 and BRCA2 were identified. However, recent studies have suggested that mutations in these genes are associated with a smaller number (20 to 60%) of hereditary breast cancer families than originally estimated, especially in studies that have been based on population-based family materials. Several groups including ours are searching for additional breast cancer susceptibility genes using whole genome scanning approaches, but the success of many of these approaches depends on the underlying heterogeneity of the remaining cancer susceptibility loci. The failure to date to identify additional breast cancer susceptibility genes associated with a high risk of disease suggests that more than one may exist. We have taken the approach that the next BRCA genes will be those that encode for proteins whose functions are linked to important cell regulatory pathways. We have recently found one such candidate BRCA3 protein, referred to as BRCC36.

14. SUBJECT TERMS
Breast cancer susceptibility, BRCA1 interacting protein

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION:

The strongest known epidemiological risk factor for breast cancer is a positive family history and studies of breast and ovarian cancer patients and their relatives consistently find statistical evidence for involvement of autosomal dominant genes. As a result of the cloning of two prominent breast-ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, clinical screening of women from high-risk families for germ-line mutations has been realized. Mutations in these two breast cancer susceptibility genes accounts for most multiple-case breast cancer families containing members with ovarian cancer or male breast cancer (1, 2). However, mutations in the *BRCA1* and *BRCA2* genes have been identified in only about 40% of families with four or five cases of female breast cancer but not cases of male breast cancer (1, 2). Several groups are searching for these genes by use of whole genome scanning, but the success of this approach depends on the underlying heterogeneity of the remaining cancer susceptibility loci. Furthermore, these suspected breast cancer families are too numerous to be statistical "accidents" of non-hereditary breast cancer. Therefore, the failure to date to identify additional breast cancer susceptibility genes associated with a high risk of disease suggests that more than one may exist. We have taken the approach that the next *BRCA* genes will be those that encode for proteins whose functions are linked to those of BRCA1 and BRCA2 (e.g., associate with BRCA1 and/or BRCA2 to form large functional protein complexes). We have recently found one such candidate BRCA3 protein, referred to as BRCC36.

OVERVIEW:

We hypothesize that proteins whose functions are linked to those of BRCA1 and/or BRCA2 are likely to contribute to the remaining breast cancer susceptibility. Furthermore, we believe, based on lack of success of genome wide association studies to uncover tumor suppressor type susceptibility genes that mutations in a proto-oncogene may also predispose to breast cancer. A survey of the 30+ genes identified that have been associated with various hereditary cancer syndromes find that at least four are oncogenes (i.e., *RET*, *MET*, *KIT*, *CDK4*). Using cell lines expressing a stable Flag-BARD1, the BRCA1-associated RING domain protein, a novel E3 ubiquitin ligase complex termed BRCC was isolated containing eight polypeptides including BRCA1, BRCA2 and RAD51 (Dong et al., Molecular Cell, 2003). BRCC not only displays increased association with p53 following DNA damage but also ubiquitinates p53 *in vitro* (Dong et al., Molecular Cell, 2003). Importantly, one of these proteins, BRCC36, appears to be a novel component of the complex with sequence homology with a subunit of the signalosome and proteasome complexes. Cancer-causing truncations of BRCA1 abrogated the association of BRCC36 with BRCC (Dong et al., Molecular Cell, 2003). Moreover, BRCC36 is aberrantly expressed in a number of breast cancer tumors (42%; 8/19) and cell lines (75%; 3/4%). In comparison, only 16% (3/19) of these breast tumors showed c-*ERBB2* amplification/overexpression. Furthermore, over-expression of BRCC36 abolished BRCA1 coactivation of p53 transcriptional responsiveness. These findings identify BRCC as an ubiquitin E3 ligase complex and suggest that aberrant expression of its components, e.g., BRCC36, may contribute to various forms of breast cancer.

BODY

Progress report year 1

Task 1. Evaluate breast tumors that over-express *BRCC36* for gene amplification.

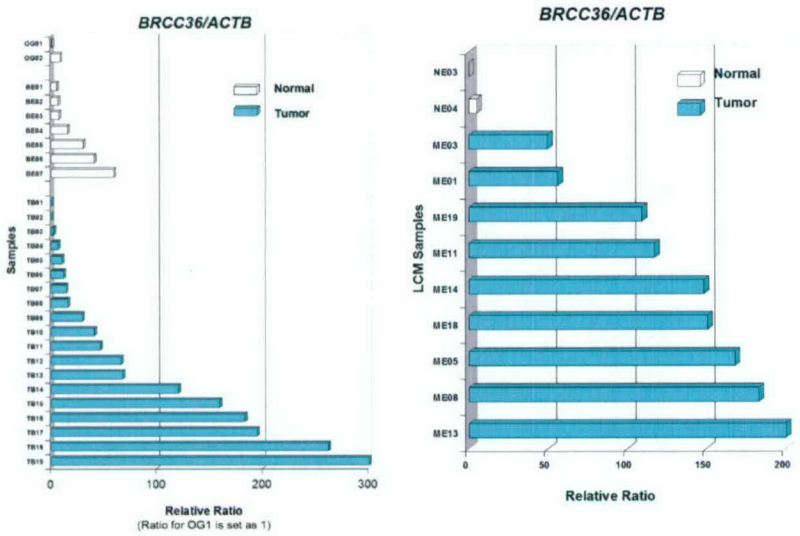

Figure 1. Aberrant mRNA expression of *BRCC36* in breast samples. (A) Quantitative PCR (QPCR) was performed to evaluate the *BRCC36* gene expression in the RNA samples isolated from breast mammary organoids (OG), primary breast epithelial cells (BE), and breast tumors (TB) *(left panel)*. **(B)** QPCR was performed to analyze normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells captured by laser micro-dissection (LCM). All levels of BRCC36 gene expression were adjusted with *beta-actin* gene expression *(right panel)*.

We have clearly shown that *BRCC36* is aberrantly expressed in the vast majority of sporadic breast tumors. Real time quantitative PCR (qPCR) was performed to evaluate the expression of *BRCC36* mRNA levels in multiple independent normal breast organoids (uncultured breast ducts composed of luminal and myoepithelial cells), primary epithelial cell cultures, non-tumorigenic breast epithelial cell lines, breast cancer cell lines, and human breast tissue specimens surgically obtained from patients with primary invasive carcinoma as described in our manuscript (3). The expression levels of *BRCC36* mRNA were elevated in 58% (11 of 19) of the breast tumors evaluated when compared to normal breast organoids (**Figure 1A**). A subset of these tumors (6 of 19) showed very high levels of expression relative to both the organoids and primary epithelial cultures (**Figure 1A**). To further validate the expression of *BRCC36* in breast tumors, we performed qPCR analysis on laser captured microdissection (LCM)-purified normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells. Two normal breast tissues and 9 invasive ductal carcinomas were microdissected and the *BRCC36* mRNA levels were evaluated by RT-PCR (**Figure 1B**). We found that 100% of these tumors (9 of 9) showed elevated levels of *BRCC36* mRNA relative to the normal mammary ductal epithelium (**Figure 1B**). We next obtained DNA from these tumors that highly expressed *BRCC36* and evaluate gene copy number by southern blotting. Gene amplification was not detected in these samples relative to normal blood DNA (data not shown). We are currently developing a PCR-based assay to more rapidly evaluate *BRCC36* gene copy number in DNA from breast tumors.

<u>Task 2.</u> Evaluate *BRCA1* and *BRCA2* mutation negative breast cancer-prone kindreds for germline *BRCC36* mutations.

We have made some progress in regards to this task and have screened a number of breast cancer

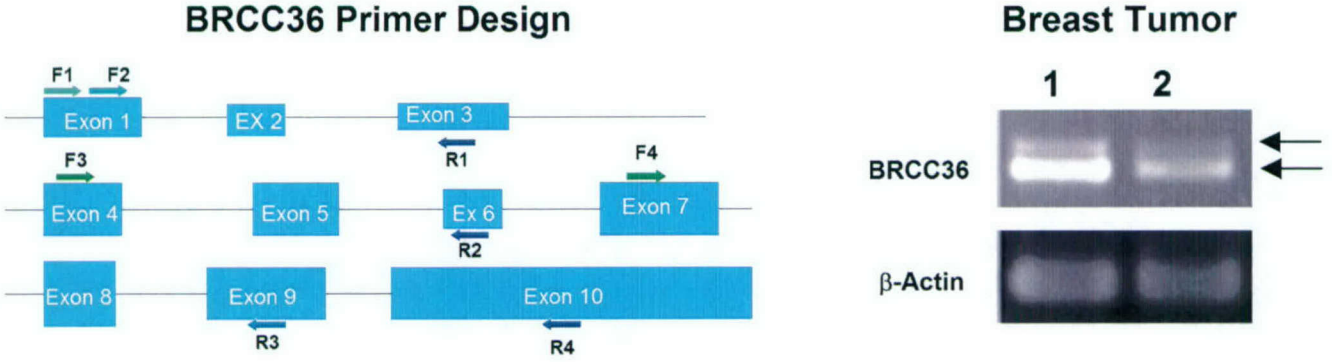

Figure 2. (A) RT-PCR primer design for *BRCC36*. A set of 4 oligonucleotide PCR primer pairs was designed to amplify the coding region of *BRCC36 (left panel)*. **(B)** RT-PCR analysis of breast tumor RNA detected a similar splice variant in *BRCC36* (*right panel*).

cell lines and cancer prone individuals for mutations in *BRCC36*. A set of 4 oligonucleotide primer pairs were designed to amplify the coding region of BRCC36 (**Figure 2A**). We first evaluated RNA by RT-PCR and direct sequencing for evidence of sequence variants in 7 breast tumor cell lines (i.e., MCF-7, MDA-MB-231, MDA-MB-435, MDA-MB-486, SK-BR-3, ZR-25-1, T-47D). No alterations were found in any of the tumor cell lines when compared to the wild-type sequence that we had established previously. We next evaluated RNA isolated from lymphoblastoid cell lines derived from women affected with breast cancer who reported a significant family history of breast and/or ovarian cancer. All had tested negative for germline mutations in *BRCA1* and *BRCA2*. Our first evaluation found no evidence for deleterious mutations in *BRCC36* in any of the 25 individuals screened. However, during these evaluations, we identified one potential splicing variants of *BRCC36* using the primer set F3/R3 (**Figure 2B**). Studies are underway to evaluate the frequency of this splice variant and determine its origin. Furthermore, we have yet to identify a sequence variant (either gemline or somatic) in *BRCC36*. This is somewhat unusual and may reflect an allele bias by evaluating only RNA. Studies are underway to evaluate DNA using high-through put methods established in the lab (4-6).

C- KEY RESEARCH ACCOMPLISHMENTS:

C.1. "BRCC36, a Novel Subunit of a BRCA1/2 E3 Ubiquitin Ligase Complex: Candidate Breast Cancer Susceptibility Gene"

1.a. Using cell lines expressing a stable Flag-BARD1, the BRCA1-associated RING domain protein, we isolated an E3 ubiquitin ligase complex termed BRCC containing eight polypeptides including BRCA1, BRCA2 and RAD51.

1.b. Reported that BRCC not only displays increased association with p53 following DNA damage but also ubiquitinates p53 *in vitro*.

1.c. Demonstrated that cancer-causing truncations of BRCA1 abrogated the association of BRCC36 with BRCC.

1.d. Reported that depletion of BRCC36 by the small interfering RNAs (siRNAs) resulted in increased sensitivity to ionizing radiation, loss of G2/M checkpoint, decreased homology-directed DNA repair and deregulation of BRCC ubiquitin E3 ligase activity.

1.e. Demonstrated that *BRCC36* is expressed at very low levels in normal breast epithelial cells and that it is highly expressed in breast tumors.

1.f. Found that expression of *BRCC36* was not apparently associated with gene amplification.

1.g. Evaluated *BRCA1* and *BRCA2* negative families for germline mutations in *BRCC36*; no deleterious mutations have been found as of yet.

1.h. Identified a novel splice variant of *BRCC36* in breast tumors.

D-REPORTABLE OUTCOMES (5/2003 to present):

***D.1.* "BRCC36, a Novel Subunit of a BRCA1/2 E3 Ubiquitin Ligase Complex: Candidate Breast Cancer Susceptibility Gene"**

1.a. Abstracts

Chen, X., Dong, Y., Hakimi, M.-A., Shiekhattar, R. and Godwin, A.K. Aberrant expression of *BRCC36*, a novel subunit of a BRCA1 E3 ubiquitin ligase complex, in sporadic breast cancer. The 95th Proceedings of the American Association for Cancer Research, abstract #4235, 2004.

1.b. Publications

Dong, Y., Hakimi, M-A., X. Chen, Kumaraswamy, E., Cooch, N.S., Godwin, A.K., Shiekhattar, R. Regulation of BRCC, a Holoenzyme Complex Containing BRCA1 and BRCA2, by a Signalosome-like Subunit and its Role in DNA Repair. Molecular Cell, 12:1087-1099, 2003.

Book chapters and review articles:

Pan, Z-Z., and Godwin, A.K. Oncogenes, Cancer, and Targeted Therapy. Life and Analytical Science, in press, 2004.

C.T. Bui, E. Nicolas, G. Sallmann, M. Chiotis, A. Lambrinakos, K. Rees, I. Trounce, R.G.H. Cotton, L. Hancock, A.K. Godwin, And Anthony T. Yeung. Enzymatic and Chemical Cleavage Methods to Identify Genetic Variation . In Molecular Diagnostics (Ed. G. Patrinos and W Ansorge) in press 2004.

E-CONCLUSIONS:

***E.1.* "BRCC36, a Novel Subunit of a BRCA1/2 E3 Ubiquitin Ligase Complex: Candidate Breast Cancer Susceptibility Gene"**

The biochemical pathways that are disrupted in the genesis of familial and sporadic breast cancers remain unclear. Moreover, the present prognosticating markers used to determine the prognosis of node-negative-patient leads to probabilistic results and the eventual clinical course is far from certain. Here we identified the human BRCC36 complex, an E3 ubiquitin ligase complex containing eight polypeptides including BRCA1, BRCA2 and RAD51. We show by LCM and real-time PCR approaches that while *BRCC36* is expressed at very low levels in normal breast epithelial cells, it is highly expressed in breast tumors. Reduction of BRCC36 levels in breast cancer cell lines by siRNA result in increased sensitivity to ionizing radiation, loss of G2/M checkpoint, decreased homology-directed DNA repair and deregulation of BRCC ubiquitin E3 ligase activity. These findings identify BRCC as an ubiquitin E3 ligase complex that enhances cellular survival following DNA damage. Although BRCC36 does not appear to be mutated in a limited series of clinical samples, studies are underway to further evaluate it role in both sporadic and familial forms of breast cancer.

F-REFERENCES:

1. Ford, D., Easton, D., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B., Gayther, S., Birch, J., Lindblom, A., Stoppa-Lyonnet, D., Bignon, Y., Borg, A., Hamann, U., Haites, N., Scott, R., Maugard, C., Vasen, H., Seitz, S., Cannon-Albright, L., Schofield, A., Zelada-Hedman, M., and Consortium, t. B. C. L. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families, Am J Hum Genet. 62*:* 676-689, 1998.

2. Bove, B, Dunbrack, R., Godwin, A.K. *BRCA1*, *BRCA2*, and Hereditary Breast Cancer. Breast Cancer: Prognosis, Treatment and Prevention. Ed. J. Pasqualini. Marcel Dekker Inc., Publisher Chapter 19, pp. 555-624, 2002.

3. Dong, Y., Hakimi, M-A., X. Chen, Kumaraswamy, E., Cooch, N.S., Godwin, A.K., Shiekhattar, R. Regulation of BRCC, a Holoenzyme Complex Containing BRCA1 and BRCA2, by a Signalosome-like Subunit and its Role in DNA Repair. Molecular Cell, 12:1087-1099, 2003.

4. Kulinski, J., Besack, D., Oleykowski, C.A., Godwin, A.K., and Yeung, A.T. CEL I enzymatic mutation detection assay. BioTechniques, 29:44-48, 2000.

5. Andrulis, I.L., Anton-Culver, H., Beck, J., Bove, B., Boyd, J., Buys, S., Godwin, A.K., Hopper, J.L., Li, F. Neuhausen, S., Ozcelik, H., Santella, R.M., Southey, M., van Orsouw, N.J., Venter, D., Vijg, J., Whittemore, A. and the CFRBCS. Comparison of methods for detection of mutations in the BRCA1 gene. Human Mutation, 20:65-73, 2002.

6. C.T. Bui, E. Nicolas, G. Sallmann, M. Chiotis, A. Lambrinakos, K. Rees, I. Trounce, R.G.H. Cotton, L. Hancock, A.K. Godwin, And Anthony T. Yeung. Enzymatic and Chemical Cleavage Methods to Identify Genetic Variation . In Molecular Diagnostics (Ed. G. Patrinos and W Ansorge) in press 2004